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Effects of the aurora kinase inhibitors AZD1152-HQPA and ZM447439 on growth arrest and polyploidy in acute myeloid leukemia cell lines and primary blasts

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ABSTRACT

Background

Aurora kinases play an essential role in the orchestration of chromosome separation and cytokinesis during mitosis. Small-molecule inhibition of the aurora kinases has been shown to result in inhibition of cell division, phosphorylation of histone H3 and the induction of apoptosis in a number of cell systems. These characteristics have led aurora kinase inhibitors to be considered as potential therapeutic agents.

Design and Methods

Aurora kinase gene expression profiles were assessed in 101 samples from patients with acute myeloid leukemia. Subsequently, aurora kinase inhibitors were investigated for their *in vitro* effects on cell viability, histone H3 phosphorylation, cell cycle and morphology in acute myeloid leukemia cell lines and primary acute myeloid leukemia samples.

Results

The aurora kinase inhibitors AZD1152-HQPA and ZM447439 induced growth arrest and the accumulation of hyperploid cells in acute myeloid leukemia cell lines and primary acute myeloid leukemia cultures. Furthermore, both agents inhibited histone H3 phosphorylation and this preceded perturbations in cell cycle and the induction of apoptosis. Single cell cloning assays were performed on diploid and polyploid cells to investigate their colony-forming capacities. Although the polyploid cells showed a reduced capacity for colony formation when compared with their diploid counterparts, they were consistently able to form colonies.

Conclusions

AZD1152-HQPA- and ZM447439 are effective apoptosis-inducing agents in acute myeloid leukemia cell lines and primary acute myeloid leukemia cultures. However, their propensity to induce polyploidy does not inevitably result in apoptosis.

Key words: aurora kinase, leukemia, cell cycle, hyperploid.

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The online version of this article contains a supplementary appendix.

Introduction

The aurora kinase family of serine/threonine proteins regulates many processes during cell division.¹⁻⁴ Accurate mitotic chromosome separation is essential for maintaining the correct ploidy of cells. Three forms of aurora kinase exist, aurora kinases A, B and C; all have a highly conserved kinase domain but differ in their N-terminal regions, which vary in length and show little or no similarity.⁵ Proliferating cells express aurora kinases A and B and these are overexpressed in tumor cells; furthermore, gene amplification of aurora kinase A has been reported in breast, ovarian, colon, prostate, neuroblastoma and cervical cancer cells.⁶⁻¹⁰ Overexpression resulted in centrosome amplification, chromosomal instability and transformation in mammalian cells.^{8,11} The aurora kinase A family functions in centrosome separation and localizes at the centrosomes, spindle poles and spindle microtubules, in prophase to telophase.^{4,6} Aurora kinase A protein levels and kinase activity are both increased in the late G₂-M phase.¹ In diploid cells, aurora kinase B localizes to the centromere in prophase and metaphase, then transfers to the cortex and spindle mid-zone in anaphase. Aurora kinase B is localized to the compacted mid-zone in telophase.¹² Histone H3 phosphorylation is important for chromosome condensation and is controlled by aurora kinases A and/or B.^{5,6,13} Inhibition of histone H3 phosphorylation has been reported to prevent initiation of chromosome condensation and entry into mitosis, implying that phosphorylation of histone H3 is essential for entry into mitosis,¹⁴ although other reports have shown progression of cells through mitosis with normal kinetics in the absence of histone H3 phosphorylation.¹⁵ Ribonucleic acid interference (RNAi) of aurora kinase A expression in the HeLa cell line impaired entry to mitosis, although DNA replication was unaffected. RNAi-targeted cells are delayed in the G₂ phase.³ RNAi inhibition of aurora kinase A, B or both in HeLa, Calu6 and U2OS cells showed that lack of aurora kinase A in these cells resulted in an increase in mitotic index, elevated cyclin B1 levels and spindle defects. Prevention of the function of aurora kinases A and B by treatment with dual inhibitors resulted in a phenotype resembling that caused by the inhibition of aurora kinase B, with DNA endoreduplication in the absence of cytokinesis.¹⁶ Several novel small-molecule inhibitors of the aurora kinases have been developed and have all shown similar effects on cell lines.¹³ The aurora kinase inhibitors ZM447439, AZD1152, VX-680 and hesperadin all resulted in inhibition of histone H3 phosphorylation and cell division. The effects of prolonged exposure were dependent on the cell lines, with some exhibiting massive polyploidy during progression through additional cell cycles without division, while others showed arrest in a pseudo G₁ state or apoptosis.^{13,15,17,18} The aurora kinases have an ATP-binding site with an adjacent cleft, which is not present in other kinases. ZM447439 binds both the ATP-binding site and the adjacent cleft thus giving this inhibitor its specificity for aurora kinases. The sequence homology between aurora kinases A, B and C has suggested that they are inhibited by ZM447439 through the same mechanism.^{15,19} A panel of cell lines (A549, DLD-1, U2OS and HeLa) that had been treated with ZM447439 accumu-

lated with 4N DNA content and undetectable histone H3 phosphorylation.¹⁵ Treatment of 3T3 cells, *Xenopus* eggs or egg extracts with ZM447439 resulted in inhibition of histone H3 phosphorylation and polyploidy.^{20,21} Previous studies have reported an IC₅₀ value for ZM447439 of 1000 nM for aurora kinase A and 50 nM for aurora kinase B.²² AZD1152 was shown to be effective on SW620 tumors in nude mice after a single dose resulted in significant and durable inhibition of tumor growth; as a result this compound has been selected for clinical evaluation.²³ AZD1152 is 1000-fold more selective for aurora kinase B than for aurora kinase A with Ki values of 1300 nM and 0.36 nM for aurora kinase A and B, respectively.²⁴

The aim of this study was to assess the level of expression of the aurora kinases in primary AML blasts and to determine the effects of two aurora kinase inhibitors, AZD1152-HQPA and ZM447439, on myeloid cell lines and primary samples from patients with AML.

Design and Methods

Cell culture

Cell lines were maintained in their appropriate medium. The NB4 and NB4R2 cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium (Sigma UK Ltd., Poole, UK) with 10% fetal bovine serum (FBS; Invitrogen Ltd, Paisley, UK). The HL60, K562 and U937 cell lines were maintained in RPMI, 10% FBS, and 1% penicillin/streptomycin (Sigma UK Ltd.). The KG1 cell line was maintained in Iscove's modified Dulbecco's medium (IMDM; Sigma UK Ltd.), 20% FBS and 1% penicillin/streptomycin. The cells were kept at concentrations between 2×10⁵ and 1×10⁶/mL. Bone marrow and venous blood samples were collected in preservative-free heparin or EDTA from newly diagnosed AML patients entering the United Kingdom Medical Research Council (UK MRC) AML14 and AML15 trials at hospitals throughout the UK. Primary cells extracted from bone marrow or peripheral blood of AML patients enriched by density gradient centrifugation with Histopaque (Sigma UK Ltd.) were maintained in RPMI medium with 10% FBS and 1% penicillin/streptomycin. All cultures were kept at 37°C, 5% CO₂. Human samples involved in this study were donated following written informed consent using documentation approved by the Medical Research Ethics Committee and the institutional review board of the University Hospital of Cardiff, Wales.

Cell imaging

Cytospin preparations of cell lines treated with the aurora kinase inhibitors at 0.01 μM were prepared, stained with modified Wright's stain and viewed by light microscopy.

Cell viability and cell counting

Cell viability was measured by trypan blue exclusion, and the expansion of cultures was quantified by manual cell counting using a hemocytometer on sequential days in culture following treatment of cell lines with AZD1152-HQPA and ZM447439 at concentrations of 1.0, 0.1 and 0.01 μM.

Annexin V positivity

Annexin V positivity was determined in treated cells using the Alexis Biochemicals Annexin V-FITC Apoptosis Detection Kit (Axxora (UK) Ltd., Nottingham, UK) according to the manufacturer's instructions. Briefly, following treatment for 48 hours with the inhibitors the cells were washed in phosphate-buffered saline (PBS) and resuspended in the supplied binding buffer containing calcium chloride and incubated with annexin V-fluorescein-isothiocyanate (FITC) in the dark for 10 minutes. Untreated samples were also prepared in this manner. Cells were then washed with PBS and resuspended in the supplied binding buffer and 1 µg/mL propidium iodide (Sigma) added. Data on the annexin V positivity of the cells were collected on a FACScalibur and analyzed using WinMDI software.

Histone H3 phosphorylation status and cell cycle analysis

The phosphorylation status of histone H3 was determined by flow cytometry. Cell lines and primary cells were treated with 0.1 µM and 0.01 µM concentrations of both inhibitors for 24 and 48 hours alongside untreated control cultures of the same cells. After 24 and 48 hours the cells were harvested by centrifugation, washed in PBS, resuspended by vortexing in ice-cold 70% ethanol and stored at -20°C until analysis. Cells were rehydrated with 0.5 mL PBS (Invitrogen Ltd.) with 1% bovine serum albumin (PBS/BSA; Sigma) and centrifuged to remove residual ethanol. Cells were then resuspended in 200 µL PBS/BSA with 0.25% Triton X100 (Sigma) and incubated on ice for 20 minutes. A washing step with 0.5 mL PBS/BSA was performed and the cells were incubated overnight in 400 µL PBS/BSA and anti-phospho histone H3 antibody (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) at 4°C. Following the overnight incubation, cells were washed with PBS/BSA and incubated with FITC-goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS/BSA for 1 hour in the dark at 4°C. Cells were washed with PBS/BSA and resuspended in 50 µL (10 µg/mL) propidium iodide and 10 µg/mL RNase (Sigma). Fluorescence data on the cells were collected using a FACScalibur (BD UK Ltd., Cowley, Oxfordshire, UK) and analyzed using WinMDI software. Cell cycle analysis was performed using Cylchred software. This software was used to determine the percentage of cells in the sub-G₁ (<G₁), G₁, S, G₂, and greater than G₂ (>G₂) regions of the cell cycle.

Gene expression analysis

Expression levels of aurora kinases A, B and C were validated by quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) which was performed on 101 patients' samples. Primers used for RQ-PCR were:

AuroraAR 5' ACTGACCACCCAAAATCTGC 3'
 AuroraAL 5' TGGAATATGCACCACTTGG 3'
 AuroraBR 5' GGCGATAGGTCTCGTTGTGT 3'
 AuroraBL 5' GGGAGAGCTGAAGATTGCTG 3'
 AuroraCR 5' ACCAGCCAAAATCTTGCAATC 3'
 AuroraCL 5' GAATATGCTCCAAGGGGTGA 3'

Clonogenicity assays in treated cell lines

AML cell lines were treated with the aurora kinase inhibitors (0.01 µM) for 48 hours before being washed and re-plated into fresh medium. Polyploid cells were distinguished on the basis of their forward scatter and side light scatter characteristics; being larger than diploid cells in the untreated cell populations derived from the same cell line. Clumps of cells were discriminated from genuine polyploidy by using pulse width (time of flight) through the laser beam. Polyploid cells were sorted such that one cell was introduced into each well of a 96-well plate. Untreated cells of each cell line were also sorted in the same manner to act as a control for any effects of the cell sorting process. The treated and untreated control cells placed into the 96-well plates were incubated at 37°C in 5% CO₂ for 7 days before an assessment of colony formation (>50 cells) or clusters (>5 cells, <50 cells) by light microscopy.

Results

Expression of aurora kinases in primary AML samples

The relative levels of aurora kinases A, B and C were determined by RQ-PCR on RNA extracted from the myeloid cell lines and 101 samples from patients with AML; only 51 samples were analyzed for aurora kinase C (Table 1). Aurora kinase C was shown to be absent in all cell lines and patients' samples tested. Aurora kinase A and aurora kinase B were expressed in all cell lines, with the expression in HL60 cells being double that in KG1 (the cell line with the lowest expression level). A wide range (>2 log difference) of expression of aurora kinase A and aurora kinase B was seen in primary AML samples. Nearly 10% of patients had significant expression of aurora kinase B (>0.01 relative to the control S14 gene). Aurora kinase A gene expression indicated that this isoform was more widely expressed in primary AML samples (~37%) and there were clear correlations with FAB groups; the majority of samples (92%) from patients with M3 AML were positive for aurora kinase A expression, whereas most samples (85%) from patients with M4 AML were negative for aurora kinase A expression (Table 1). No correlation with age or sex was observed. Expression of aurora kinases A and B was also determined using the Affymetrix gene expression system in normal donor bone marrow samples and in a panel of AML cell lines. These data are shown in *Online Supplementary Figure S1*. There was good correlation between the results achieved using RQ-PCR and the Affymetrix system.

Morphological effect aurora kinase inhibitors on cell lines

The effect of treating cell lines with 0.01 µM AZD1152-HQPA and ZM447439 for 48 hours on the morphology of the cells was observed by light microscopy and digital images of the cells were recorded (Figure 1A, B, C). Treatment with both aurora kinase inhibitors resulted in a visible increase (approximately doubling) in cell size when compared to untreated cells at 48 hours, with clear morphological evidence of apoptosis in the majority of

Table 1. Gene expression levels of aurora kinases in AML patients as determined by quantitative RT-PCR.

	Number analyzed	>0.01 relative expression	% expressed	p value
Aurora kinase A	101	37	37	
M1	22	10	42	
M2	23	7	30	
M3	11	10	92	$p < 0.0001$ compared to non-M3
M4	26	4	15	$p < 0.01$ compared to non-M4
M5	6	2	33	
Others	13	4	31	
Aurora kinase B	101	10	10	
Aurora kinase C	51	0	0	

cells. A more quantitative assessment of the changes in the untreated and treated cells was obtained using flow cytometry. AZD1152-HQPA-treated cells showed size increases in a time-dependent manner assessed by increase in forward light scatter as an index of increasing cell size (NB4 cells shown in Figure 1D, E, F). The presence of the large cells in the cultures was accompanied by an increased level of cellular debris, indicating that the cells were undergoing apoptosis.

Effect on cell cycle

The effect of the aurora kinase inhibitors on the cell cycle was determined by flow cytometry (Figure 2) following treatment with AZD1152-HQPA and ZM447439 at 1.0 μM (cell lines only), 0.1 μM and 0.01 μM for 48 hours. The treated cell lines (all data shown in *Online Supplementary Table S1*) consistently showed an increase in the percentage of cells with a DNA content of 4N and >4N following treatment with AZD1152-HQPA, with concurrent decreases in the percentage of cells with a DNA content of 2N. Following treatment with AZD1152-HQPA there were significant increases in the >4N region (0.01 μM AZD1152-HQPA; $p=0.007$) and in the 4N region (0.1 μM AZD1152-HQPA; $p=0.012$) and decreases in the percentage of cells in 2N (0.01 μM

AZD1152-HQPA; $p=0.043$). Figure 2A shows the change in the DNA content of cell lines treated with AZD1152-HQPA for 48 hours. Treatment of primary AML cells with AZD1152-HQPA resulted in less noticeable perturbations of the cell cycle (Figure 2B). Analysis of primary samples treated with AZD1152-HQPA (*Online Supplementary Table S2*) showed significantly fewer cells with a 2N DNA content (0.01 μM , AZD1152-HQPA; $p=0.039$) and increased numbers of cells in S phase (0.01 μM , AZD1152-HQPA; $p=0.033$; Figure 2B).

Treatment with ZM447439 resulted in a reduced percentage of cells with 2N content (40.9% to 24.5% (1.0 μM , ZM447439); $p<0.0001$) and an increased percentage of cells with >4N DNA (1.7% to 3.7% (0.01 μM , ZM447439; $p=0.026$, although 1.0 μM ZM447439 resulted in 18.2% of cells having >2N DNA content) (*Online Supplementary Table S1*). Analysis of primary samples (*Online Supplementary Table S2*) treated with ZM447439 (0.01 μM) also produced a decrease in cells with 2N DNA content (55.7% to 49.80%, $p=0.010$) and increased proportions in S phase (24.8% to 30.7%, $p=0.012$). Increases in the 4N and >4N regions of the cell cycle were observed but were not statistically significant (Figure 2B).

Effect of AZD1152-HQPA and ZM447439 on the proliferative capacity of cell lines

AZD1152-HQPA, at concentrations as low as 0.01 μM , produced a significant ($p\leq 0.05$) decrease in the ability of NB4 cells to proliferate by 48 hours (Figure 3). The other cell lines (HL-60, NB4R2, K562, KG1 and U937) also showed reduced proliferative capacity following treatment with AZD1152-HQPA. The viability of the NB4 cells was decreased at 48 hours by treatment with 0.01 μM AZD1152-HQPA ($p\leq 0.05$). The other cell lines also showed decreases in viability following treatment with AZD1152-HQPA. ZM447439 induced a significant decrease in the proliferative capacity at the higher concentration of 1.0 μM in NB4 cells after 24 hours (Figure 3) and the viability of NB4 cells was reduced after 48 hours with 1.0 μM ZM447439. Similar effects were seen on the other cell lines (NB4R2, KG1,

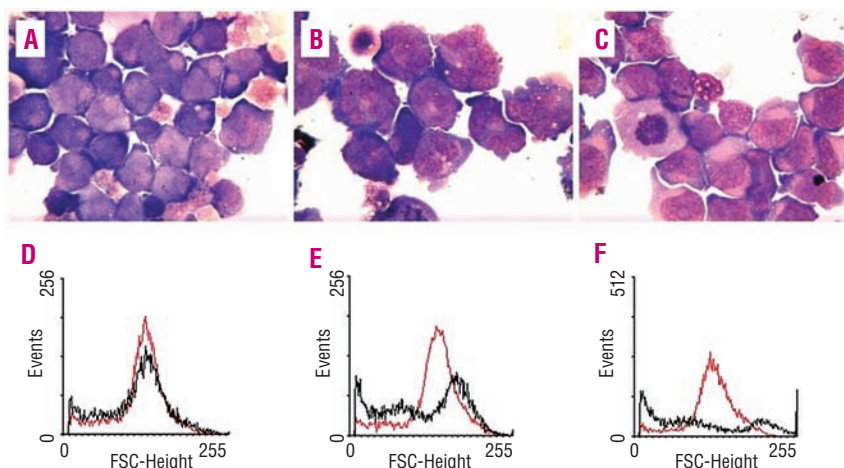


Figure 1. Morphological evidence of the changes in NB4 cells as a result of treatment with aurora kinase inhibitors. (A) Untreated NB4 cells. (B) NB4 cells treated with AZD1152-HQPA (0.01 μM) for 48 hours and (C) NB4 cells treated with ZM447439 (0.01 μM) for 48 hours. All images are at 40X magnification. Sizes of untreated NB4 cells (red) and cells treated with 0.01 μM AZD1152-HQPA (black) for (D) 24 hours, (E) 48 hours and (F) 72 hours measured by forward light scatter using flow cytometry. The same pattern of increasing cell size with treatment was only observed when NB4 cells were treated with ZM447439 at 1.0 μM , while lower concentrations had no effect on cell size. Data are representative of three repetitions of this experiment.

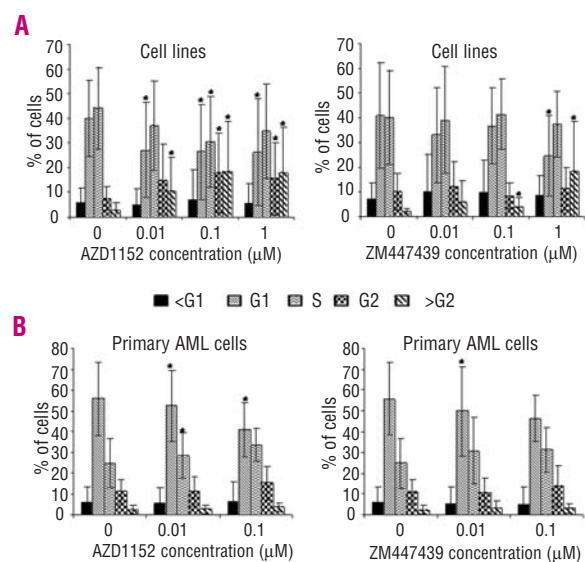


Figure 2. The effect on cell cycle resulting from treatment with varying concentrations of aurora kinase inhibitors for 48 hours was assessed using cychred software. (A) The distribution of six myeloid cell lines assayed in triplicate and (B) primary cells from 30 AML patients treated with the indicated concentrations of AZD1152-HQPA and ZM447439 are shown in the histograms. *Denotes a change in cell cycle distribution that is significantly different from that in the untreated population ($p < 0.05$) measured using a paired t test. Error bars indicate \pm one standard deviation.

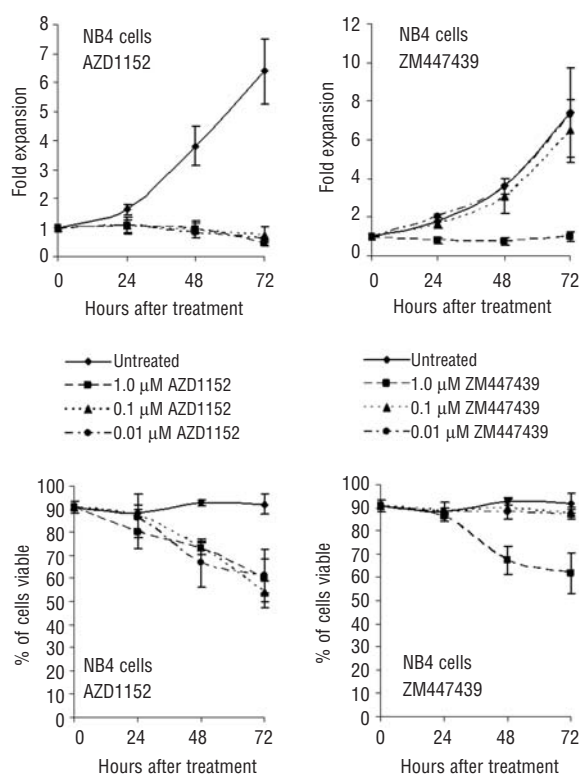


Figure 3. NB4 cells were treated with concentrations of AZD1152-HQPA and ZM447439 between 1.0 μM and 0.01 μM for 72 hours. The growth and viability of the cells were recorded at 24 hour intervals to assess the effect of the aurora kinase inhibitors on the cells. Experiments were repeated in triplicate. All concentrations of AZD1152-HQPA used proved to be effective at reducing the growth and viability of the NB4 cells while only the highest concentration of ZM447439 resulted in the same effect on the NB4 cells.

K562, HL60 and U937), which also showed reduced proliferation following treatment with ZM447439. Annexin V expression was measured in NB4 cells following treatment with the inhibitors for 48 hours. The results presented in *Online Supplementary Figure S2* show that apoptosis was induced in the cells in a concentration-dependent manner.

Effect of AZD1152-HQPA and ZM447439 on phosphorylation of histone H3

Histone H3 phosphorylation on serine 10 was measured by flow cytometry following treatment of cell lines and primary AML samples with the aurora kinase inhibitors for 24 and 48 hours at a range of concentrations. Figure 4A illustrates the gating strategy used in order to identify cells with phosphorylated histone H3. The level of phosphorylated histone H3 detectable in untreated cell lines was low (approximately $2.1 \pm 1.89\%$ of cells showed phosphorylation) and even lower in the primary samples (1%). Treatment of cell lines with AZD1152-HQPA for 24 or 48 hours produced significant decreases in the level of phosphorylated histone H3 present in the cells (Figure 4B). After 24 hours of treatment with 0.1 μM AZD1152-HQPA, histone H3 was reduced to 63% of the level in untreated controls ($p = 0.029$) with a further reduction observed after 48 hours of treatment to 39% of untreated levels ($p = 0.044$). Primary AML cells treated with 0.1 μM AZD1152-HQPA (Figure 4B) showed a decrease in histone H3 phosphorylation after 24 hours to 78% of the control level ($p < 0.0001$) and after 48 hours 0.01 μM AZD1152-HQPA resulted in reduction to 63% of the control level ($p = 0.043$). In cell lines, the effect of 48 hours of treatment with ZM447439 was a significant decrease in phosphorylation to 60% of the control level ($p = 0.006$). In primary samples from AML patients, 0.1 μM ZM447439 inhibited phosphorylation of histone H3 to 58% of the control level by 48 hours ($p < 0.0005$).

Correlation between expression of aurora kinases and response to inhibitors

To determine whether the primary cells expressing aurora kinases A and B had an increased response to the inhibitors, samples expressing and not expressing aurora kinases were assessed for their responses to treatment with the inhibitors. A response was defined as an increase in annexin V positivity, a decrease in histone H3 phosphorylation or an increase in the percentage of cells with a $>4N$ DNA content after 48 hours of treatment. An increase in annexin V expressing cells was seen in 57% of aurora kinases A-expressing cells, 67% of aurora kinases B-expressing cells and in 58% of cells that did not express either aurora kinases A or B. Histone H3 phosphorylation was seen in 50% of cells expressing aurora kinase A or B and in 58% of the cells that did not express either aurora kinase. An increase in the percentage of cells with $>4N$ DNA was seen in 50% of the aurora kinase A-expressing samples, in 67% of the aurora kinase B-expressing samples and in 50% of the samples that did not express either aurora kinase. From these results it is clear that there is no correlation between aurora kinase expression and response to aurora kinase inhibition.

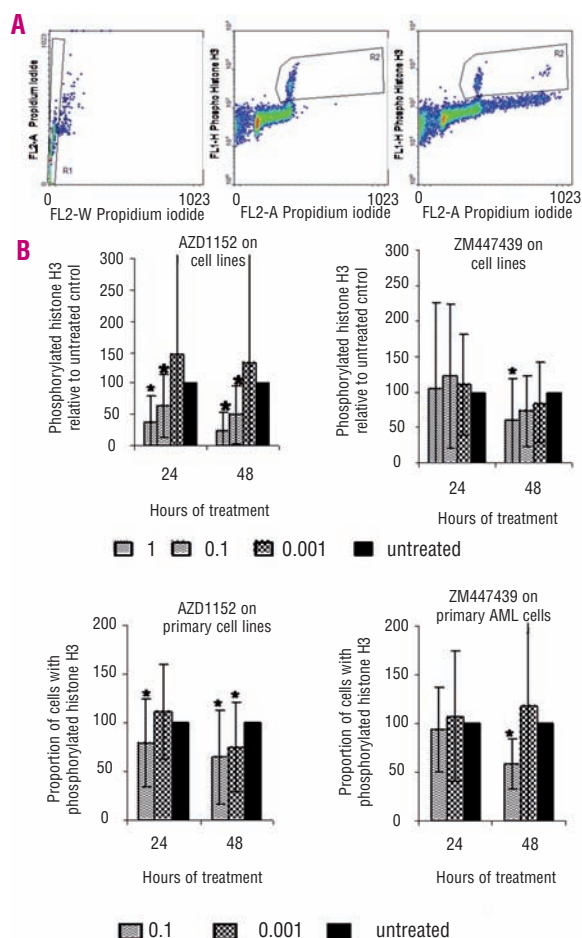


Figure 4. The effect of treatment with AZD1152-HQPA and ZM447439 on phosphorylation of histone H3 in NB4 cells. (A) Flow cytometry was used to determine the percentage of cells with phosphorylated histone H3. Doublets were removed from the analysis using the R1 gate shown in the first plot. The remaining plots show propidium iodide staining, used to demonstrate the DNA content of the cells against the level of phosphorylated histone H3 for each cell measured. At 48 hours, the untreated population had 2.45% phosphorylated histone H3 (center) and AZD1152-HQPA-treated cells (0.01 μM) 2.08% (right) shown in the R2 region on these plots. (B) The effect of different concentrations of AZD1152-HQPA and ZM447439 over 48 hours on a range of six cell lines and primary cells from 30 AML patients was determined by flow cytometry. A significant difference ($p < 0.05$) in phosphorylation state compared to that of the untreated control population is indicated by * and was determined using paired *t* tests.

Clonogenic potential of aurora kinase-induced polyploid cells

Polyploid cells induced by a single 48-hour exposure to aurora kinase inhibitors (0.01 μM) were isolated by high-speed cell sorting directly into 96-well plates containing culture media (one cell per well). Cell lines treated with AZD1152-HQPA or ZM447439 showed reduced colony-forming ability compared with that of the untreated control cells. The average colony growth across all the cell lines was reduced from 391 colonies to 134 colonies when the cells were treated with AZD1152-HQPA ($p = 0.003$) and to 154 colonies when they were treated with ZM447439 ($p = 0.022$). The numbers of colonies grown following treatment and re-plating in fresh medium are shown in Figure 5.

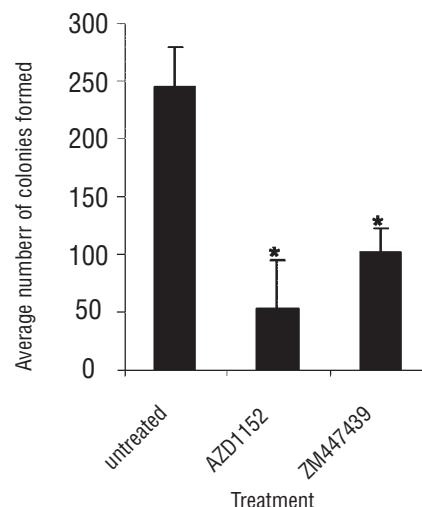


Figure 5. The effect of plating six myeloid cell lines (HL-60, U937, NB4, NB4R2, KG1 and K562) treated with aurora kinase inhibitors AZD1152-HQPA and ZM447439 (0.01 μM) for 48 hours into fresh medium was assessed by counting the number of colonies of cells that had grown after 1 week from cells plated into culture dishes as single cells per well. The experiments were repeated in triplicate and statistical significance was determined using a paired *t* test: * $p = 0.003$ and * $p = 0.022$ for AZD1152-HQPA and ZM447439 compared to untreated, respectively.

Discussion

Depending on their age, 50-70% of adults with AML achieve a complete remission but only 20-30% of these experience long-term disease-free survival with conventional chemotherapy.²⁵ Improvements in clinical outcomes, particularly in patients over 55 years old, have been modest,²⁵ as these patients do not tend to tolerate intensive chemotherapeutic regimens and frequently have poor prognosis cytogenetics.²⁷ Overall survival rates in older patients are less than 10%.^{25,26} This inability to successfully treat older patients with AML underlies the continuing need to develop new treatments for AML.

This study is the first report of aurora kinase expression levels in patients with AML. We found that aurora kinase A was expressed in 37% of primary AML samples, while aurora kinase B was only expressed in 10% of such samples. The low mitotic rate of AML cells in *in vitro* culture may be one reason why only some of the population were found to express these kinases. Interestingly, there appeared to be a correlation between aurora kinase expression and FAB group, with the M3 subtype predominantly exhibiting aurora kinase A expression, while the M4 subtype usually had very low expression of both aurora kinase A and aurora kinase B. The expression of the aurora kinases in a range of cancers (breast, ovarian, colon, prostate, neuroblastoma and cervical cancer cells)⁶⁻¹⁰ in which expression was correlated with tumor progression suggested that aurora kinases may be a potential target for treatment. However, given the levels of expression in primary AML cells, probably only a sub-group of patients might be responsive to aurora kinase therapy.

Although no correlation was seen between expression of the aurora kinases and response to their inhibition, there are various possible explanations for this. Our favored hypothesis is that a critical level of aurora kinase expression is required to maintain cell viability, meaning that cells with lower aurora kinase expression may be more susceptible to aurora kinase inhibition; this hypothesis does, however, need to be tested experimentally. Proliferating cells express aurora kinase A and B and aurora kinase A is required for the commitment of human cells to mitosis.³ Most previous studies^{3,15-17,28-30} were conducted on solid tumors and not on circulating blast cells. The effects of inhibiting the aurora kinases in different types of cancer suggest that aurora kinase inhibitors trigger apoptosis in a proportion of cells and lead to arrest of tumors in model systems.¹⁸ In our studies, both AZD1152-HQPA and ZM447439 resulted in a decreased proliferative capacity and viability of AML cell lines. This agrees with the findings of previous studies in which ZM447439 inhibited proliferation completely and resulted in endoreduplication in human cell lines.¹⁵ Another aurora kinase inhibitor, VX680, also inhibited proliferation and leukemic cells were found to be particularly sensitive to this inhibitor.¹⁷

Treatment of the cell lines and primary AML material with the inhibitors AZD1152-HQPA and ZM447439 resulted in the disruption of the cell cycle with accumulation of cells with 4N and >4N DNA content, alongside a decrease in the proportion of cells with <4N DNA content. In agreement with the data described here, ZM447439 has previously been reported to result in accumulation of cells with 4N DNA content in a variety of human cell lines (A549, MCF-7, DLD-1, HeLa and U2OS).¹⁵ There was also an accumulation of polyploid cells as a result of treatment with VX-680.^{5,17}

Histone H3 phosphorylation is controlled by the aurora kinases and is important for chromosome condensation.^{5,6,13} When histone H3 phosphorylation is inhibited, chromosome condensation is prevented and entry to mitosis blocked. Histone H3 phosphorylation was measured as a means of determining whether the aurora kinase inhibitors AZD1152-HQPA and ZM447439 had an effect on the cells. However, the level of histone H3 phosphorylation seen in the primary AML cells was low (~2%) even in the absence of inhibitors. In our study, phosphorylation of histone H3 was reduced in cell lines after treatment with AZD1152-HQPA and ZM447439. This confirms previous reports that ZM447439 treatment of human cell lines and *Xenopus* eggs resulted in loss of phosphorylation of histone H3.^{15,21} Histone H3 phosphorylation was also inhibited in the human cell line MCF-7 and rat cell line PtK₂ after treatment with alternative aurora kinase inhibitors, VX-680 and hesperadin, respectively.^{17,28} These results indicate that the aurora kinases are indeed inhibited by the aurora kinase inhibitor compounds as histone H3 phosphorylation is a direct downstream target of aurora kinase B. Our results extend these findings, showing that the aurora kinases were inhibited in both cell lines and primary AML samples.

We have shown that cell lines treated with the inhibitors for 48 hours, assessed by size and granularity to be part of the hyperploid population, showed a

reduced capacity to undergo clonal growth when re-plated as single cells in fresh medium, indicating that recovery from hyperploidy was impaired. This suggests that the majority of hyperploid cells produced as a result of treatment with AZD1152-HQPA or ZM447439 are not capable of recovering once they have reached a certain stage or DNA content and that they cannot continue to proliferate. This result is in agreement with previous findings that treatment with ZM447439 for 24 hours followed by re-plating in fresh medium without ZM447439 resulted in a dramatic reduction of the number of colonies formed.²² Another report found that continuous treatment with VX-680, which inhibits FLT3 as well as the aurora kinases, completely ablated the colony-forming ability of primary cells from AML patients with FLT3 mutations, although this probably reflects the effect of the agent on proliferation rather than the ability of cells to recover from exposure to the treatment agent once it has been removed.¹⁷

Other studies have also shown that the effect of inhibiting the action of aurora kinases is reversible. The phosphorylation of histone H3 was restored within 30 minutes after removal of hesperadin from PtK₂ cells.²⁸ When SU6668, which inhibits aurora kinase A amongst other kinases, was washed out of HeLa cells, the cells were able to re-enter the G₁ phase. Phosphorylation of histone H3 on serine 10, thought to be a measure for aurora kinase activity, was also completely abolished by the action of SU6668 but recommenced within 30 minutes of removal of this inhibitor.³⁰ These findings suggest that targeting aurora kinases may prove challenging in the setting of clinical hematology as the inhibitors may have to be given at frequent intervals or even continuously to maintain their effect. The aurora kinase inhibitor AZD1152-HQPA may have potential to treat specific sub-groups of leukemic cells with overexpression of aurora kinase A. In particular, the M3 sub-type of AML may be a specific niche for the action of these inhibitors. However, this study suggests that further investigation of these drugs is warranted, particularly AZD1152-HQPA, which appears more effective than ZM447439 in AML cell lines and primary cells.

Authorship and Disclosures

EW: principle investigator responsible for the design of the study, data acquisition, analysis, and interpretation, drafting of the manuscript; VW: acquisition, analysis and interpretation of data relating to gene expression, reviewing the manuscript; CP: concept and design of the study, interpretation of flow cytometry data and flow cytometry aspects of the study, reviewing the manuscript and approval of the version for publication; AB: concept and design of the study, critical revisions of the manuscript and approval of the version to be published; KM: concept and design of study, interpretation of acquired data, drafting, critical review and approval of the final version of the manuscript. The authors reported no potential conflicts of interest.

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